Docket No.: 0599-0213PUS1
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent Application of:
Shigehisa WADA et al.

Application No.: 10/570,488 | Confirmation No.: 7587

Filed: November 13, 2006 | Art Unit: 1797

For: METHOD OF PREPARING SOLUTION HAVING COMPOSITION OF BIOLOGICAL COMPONENTS | Examiner: J. M. Hurst

**DECLARATION SUBMITTED UNDER 37 C.F.R. § 1.132**

Honorable Commissioner
Of Patents and Trademarks
P.O. Box 1450
Alexandria, VA 22313-1450

April 28, 2010

Sir:

I, Dr. Kazuhiro Tanahashi of Specialty Materials Research Labs., Medical Device Research Lab., Toray Industries, Inc., Japan, do hereby declare the following:

I have attached a copy of my curriculum vitae to this Declaration.

I am Research Associate, the Specialty Materials Research Labs., and have worked in this field for 19 years.

I am familiar with the above referenced patent application and the area of science dealing with protein separations. I am also well versed in the use of molecular sieves and various types of chromatography for purification, isolation and concentration of proteins.

I have read and understand the subject matter of the Office Action of February 1, 2010.

The following comments are offered in support of the patentability of the instant invention.

The Examiner states that the claimed invention is not novel over the disclosure in Demmer et al. and that the claimed invention is obvious over Demmer et al. when combined with the disclosures of Kim et al. or Comper. Specifically, the Examiner implies that module 1 of Demmer et al. is a molecular sieve (see page 3 of the Office Action, first full paragraph) and that the membrane disclosed in Kim et al. could be used in the Demmer et al. procedure to produce the claimed invention. I disagree.

Molecular sieves function by separation based on molecular weight, as evidenced by Encyclopædia Britannica and Wikipedia (see attached) and as described in the Protein Purification Wikipedia article, also attached. Such separation based on molecular weight is generally referred to as size exclusion chromatography and the molecular sieves used are frequently in either membrane-type form, gel form or packed into a column. Size exclusion chromatography is only one type of chromatographic method that is available for protein separation/purification.

Demmer et al. use an ion exchange procedure to separate albumin protein from the other components of blood serum. Ion exchange membranes separate proteins having different ionic charges even if the molecular sizes of the proteins are small enough to pass through the pore size of the membrane. For example, an anion exchange procedure conducted on blood serum would retain IgG4 (Pi = <6, mw = 146) on the membrane while the positively charged IgG1 molecules (Pi = 8-9.5, mw = 146) would pass through (see Buis et al., attached, for molecular weights and isoelectric points). Consequently, when the pores of the anion exchange separation membrane allow molecules with a mw of 146 to pass through, if the molecule has a net negative charge, it will be retained but if it has a positive charge it will be separated out.

Demmer et al. follow the an anion exchange procedure in module 1 with a cation exchange procedure in module 2. Demmer et al. does not concentrate albumin based on molecular weight in either case. That is, Demmer et al. does not use a molecular sieve as required by step (2) of the instant invention.

I also disagree that the claimed invention is obvious over a combination of Demmer et al. and Kim et al. While Kim et al remove albumin by employing fractionation with a molecular sieve, in my opinion the skilled artisan would not have a reasonable expectation of success in

simply substituting the molecular sieve used by Kim in the Demmer et al. method. This is because Kim et al. indicate that the pore size of the membrane in contact with the loaded sample solution is about 10-100kD (column 7, lines 49-54) and that the charged portion of the membrane carries negative charges. However Demmer et al. uses Sartobind Q 100 and S 100 membranes, which are strong anion and cation ion exchange membranes having a pore size of >3$\mu$m (see Sartobind membrane description, attached). The correlation between the Sartobind membrane >3$\mu$m pore size and the 10-100 kD molecular weight cut off used by Kim et al. can be calculated as follows.

The cut off molecular weight of the membrane used in Kim refers to the average molecular weight of dextran at which 90% is blocked. The radius of solute is calculated from the molecular weight of dextran according to the following formula (see US 6355730 B1, column 8, line 61-67):

Radius of solute [nm] = 0.04456 X (molecular weight of dextran molecule)$^{0.43821}$

Therefore,

| | |
|---|---|
| Radius of solute [nm] | ~ (pore size of membrane)/2 |
| 0.04456 X (100000)$^{0.43821}$ | ~ |
| 6.92 nm | ~ |
| 13.8 nm | ~ pore size of membrane |

Thus, the pore size of the membrane used in the Kim et al. reference is at most about 13.8 nm, which is drastically different from the >3$\mu$m ion exchange membrane used in Demmer et al.

This significant difference in pore size would prevent the separation which occurs in the anionic exchange portion of the Demmer et al. procedure that removes many of the "contaminants," such as the higher molecular weight components of plasma. In addition, using the Kim et al. membrane would not allow for cationic exchange in the Demmer et al. procedure.

It is also my opinion that the skilled artisan would not have any reasonable expectation of success in obtaining the claimed invention by combining the blue dye disclosed in Comper with the Demmer et al. procedure. Many times addition of dyes alter the net charge associated with protein molecules and/or otherwise affects its physical or biological properties (see, for example

Kierszenbaum et al. (1969) Analytical Biochem. 28:563-572). Consequently, the skilled artisan would expect that the addition of dyes, etc. would likely change the dynamics of the Demmer et al. system and therefore could not be counted on to produce the same effect.

To conclude, in my opinion the claimed invention is significantly different from the procedure disclosed in Demmer et al., alone or in combination with either Kim et al. or Comper.

The undersigned hereby declares that all statements made herein based upon knowledge are true, and that all statements made based upon information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

DATED: May. 25. 2010 Kazuhiro Tanahashi

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PUBLICATION:

1. Kazuhiro Tanahashi, and Antonios G. Mikos, "Cell adhesion on poly(propylene fumarate-co-ethylene glycol) hydrogels", *J. Biomed. Mater. Res.*, **62**, 558-566 (2002).
2. Kazuhiro Tanahashi, S. Jo, and Antonios G. Mikos, "Synthesis and characterization of biodegradable cationic poly(propylene fumarate-*co*-ethylene glycol) copolymer hydrogels modified with agmatine for enhanced cell adhesion", *Biomacromolecules*, **3**, 1030-1037 (2002).
3. Kazuhiro Tanahashi, and Antonios G. Mikos, "Effect of hydrophilicity and agmatine

modification on degradation of poly(propylene fumarate-*co*-ethylene glycol) hydrogels", *J. Biomed. Mater. Res. A.*, **67**, 1148-1154 (2003).

4. Kazuhiro Tanahashi, and Antonios G. Mikos, "Protein adsorption and smooth muscle cell adhesion on biodegradable agmatine modified poly(propylene fumarate-co-ethylene glycol) hydrogels", *J. Biomed. Mater. Res.*, **67**, 448-457 (2003).

5. F. Kurtis Kasper, Erin Jerkins, Kazuhiro Tanahashi, Michael A. Barry, Yasuhiko Tabata, Antonios G. Mikos, "Characterization of DNA release from composites of oligo(poly(ethylene glycol) fumarate) and cationized gelatin microspheres *in vitro*", *J. Biomed. Mater. Res. A.*, **78**, 823-835 (2006).

6. F. Kurtis Kasper, Simon Young, Kazuhiro Tanahashi, Michael A. Barry, Yasuhiko Tabata, John A. Jansen, Antonios G. Mikos, "Evaluation of bone regeneration by DNA release from composites of oligo(poly(ethylene glycol) fumarate) and cationized gelatin microspheres in a critical-sized calvarial defect", *J. Biomed. Mater. Res. A.*, 78, 335-342 (2006).

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Science & Technology
: :

# molecular sieve

ARTICLE
from the
Encyclopædia Britannica

a porous solid, usually a synthetic or a natural zeolite, that separates particles of molecular dimension. Zeolites are hydrated metal aluminosilicate compounds with well-defined crystalline structures. The silicate and aluminate groupings form three-dimensional crystal lattices surrounding cavities in which the metal ions and the water molecules are loosely held. Channels run through the entire crystal, interconnecting the cavities and terminating at the crystal surface. Upon heating, the zeolites lose their water content with little or no change in their crystal structure. The dehydrated zeolite can reversibly absorb water or other molecules that are small enough to pass through the channels or pores. The metal ions are also readily replaceable by other ionic units of similar charge and size.

Molecular sieves are used for drying gases and liquids and for separating molecules on the basis of their sizes and shapes. When two molecules are equally small and can enter the pores, separation is based on the polarity (charge separation) of the molecule, the more polar molecule being preferentially adsorbed. *Compare* gel chromatography.

# Molecular sieve

From Wikipedia, the free encyclopedia

A **molecular sieve** is a material containing tiny pores of a precise and uniform size that is used as an absorbent for gases and liquids.

Molecules small enough to pass through the pores are absorbed while larger molecules are not. It is different from a common filter in that it operates on a molecular level. For instance, a water molecule may be small enough to pass through the pores while larger molecules are not. The pores also act as a trap for the penetrating water molecules which are retained within the pores. Because of this, they often function as a desiccant. A molecular sieve can adsorb water up to 22% of its own weight.[1] The principle of absorption to molecular sieve particles is somehow similar to that of size exclusion chromatography.

Often they consist of aluminosilicate minerals, clays, porous glasses, microporous charcoals, zeolites, active carbons, or synthetic compounds that have open structures through which small molecules, such as nitrogen and water can diffuse.

Molecular sieves are often utilized in the petroleum industry, especially for the purification of gas streams and in the chemistry laboratory for separating compounds and drying reaction starting materials. The mercury content of natural gas is extremely harmful to the aluminium piping and other parts of the liquefaction apparatus - silica gel is used in this case.

Methods for regeneration of molecular sieves include pressure change (as in oxygen concentrators), heating and purging with a carrier gas (as when used in ethanol dehydration), or heating under high vacuum.

## Contents



## Adsorption capabilities[2][3]

- 3A (pore size 3 Å): Adsorbs $NH_3$, $H_2O$, (not $C_2H_6$), good for drying polar liquids.
- 4A (pore size 4 Å): Adsorbs $H_2O$, $CO_2$, $SO_2$, $H_2S$, $C_2H_4$, $C_2H_6$, $C_3H_6$, EtOH. Will not adsorb $C_3H_8$ and higher hydrocarbons. Good for drying nonpolar liquids and gases.
- 5A (pore size 5 Å): Adsorbs normal (linear) hydrocarbons to n-$C_4H_{10}$, alcohols to $C_4H_9OH$, mercaptans to $C_4H_9SH$. Will not adsorb isocompounds or rings greater than $C_4$.
- 10X (pore size 8 Å): Adsorbs branched hydrocarbons and aromatics. Useful for drying gases.
- 13X (pore size 10 Å): Adsorbs di-n-butylamine (not tri-n-butylamine). Useful for drying HMPA.

## Difference Between Molecular Sieves and Zeolite

| Molecular Sieves | Zeolites |
|---|---|
| Able to distinghuish Materials on the basis of their size | Special Class of Molecular Sieves with Aluminosilicates as skeletal composition |
| may be crystalline, non-crystalline, para-crystalline or pillared clays | they are highly crystalline materials |
| variable Framework Charge with porous structure | Anionic framework with microporous and crystalline structure |

## See also

Some of the many types of molecular sieves are:

- Activated carbon
- Desiccant
- Lime (mineral)
- Silica gel
- Zeolite

## References

1. ^ Molecular sieve,and molecular sieves study
2. ^ Fieser and Fieser, Reagents for Organic Synthesis, Vol. 1, Wiley, New York, 1967, p. 703
3. ^ Breck, D.W. J. Chem. Ed., 41, 678 (1964)

## External links

- Sieves put a lid on greenhouse gas
- molecular sieve safety
- Sorbentsystems.com

# Protein purification

From Wikipedia, the free encyclopedia

**Protein purification** is a series of processes intended to isolate a single type of protein from a complex mixture. Protein purification is vital for the characterisation of the function, structure and interactions of the protein of interest. The starting material is usually a biological tissue or a microbial culture. The various steps in the purification process may free the protein from a matrix that confines it, separate the protein and non-protein parts of the mixture, and finally separate the desired protein from all other proteins. Separation of one protein from all others is typically the most laborious aspect of protein purification. Separation steps exploit differences in protein size, physico-chemical properties and binding affinity.

## Contents



## Purpose

Purification may be *preparative* or *analytical*. Preparative purifications aim to produce a relatively large quantity of purified proteins for subsequent use. Examples include the preparation of commercial products such as enzymes (e.g. lactase), nutritional proteins (e.g. soy protein isolate), and certain biopharmaceuticals (e.g. insulin). Analytical purification produces a relatively small amount of a protein for a variety of research or analytical purposes, including identification, quantification, and studies of the protein's structure, post-translational modifications and function. Among the first purified proteins were urease and Concanavalin A.

## Strategies

Choice of a starting material is key to the design of a purification process. In a plant or animal, a particular protein usually isn't distributed homogeneously throughout the body; different organs or tissues have higher or lower concentrations of the protein. Use of only the tissues or organs with the highest concentration decreases the volumes needed to produce a given amount of purified protein. If the protein is present in low abundance, or if it has a high value, scientists may use recombinant DNA technology to develop cells that will produce large quantities of the desired protein (this is known as an expression system). Recombinant expression allows the protein to be tagged, e.g. by a His-tag, to facilitate purification, which means that the purification can be done in fewer steps. In addition, recombinant expression usually starts with a higher fraction of the desired protein than is present in a natural source.

An analytical purification generally utilizes three properties to separate proteins. First, proteins may be purified according to their isoelectric points by running them through a pH graded gel or an ion exchange column. Second, proteins can be separated according to their size or molecular weight via size exclusion chromatography or by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) analysis. Proteins are often purified by using 2D-PAGE and are then analysed by peptide mass fingerprinting to establish the protein identity. This is very useful for scientific purposes and the detection limits for protein are nowadays very low and nanogram amounts of protein are sufficient for their analysis. Thirdly, proteins may be separated by polarity/hydrophobicity via high performance liquid chromatography or reversed-phase chromatography.

Recombinant bacteria can be grown in a flask containing growth media.

## Evaluating purification yield

The most general method to monitor the purification process is by running a SDS-PAGE of the different steps. This method only gives a rough measure of the amounts of different proteins in the mixture, and it is not able to distinguish between proteins with similar molecular weight.

If the protein has a distinguishing spectroscopic feature or an enzymatic activity, this property can be used to detect and quantify the specific protein, and thus to select the fractions of the separation, that contains the protein. If antibodies against the protein are available then western blotting and ELISA can specifically detect and quantify the amount of desired protein. Some proteins function as receptors and can be detected during purification steps by a ligand binding assay, often using a radioactive ligand.

In order to evaluate the process of multistep purification, the amount of the specific protein has to be compared to the amount of total protein. The latter can be determined by the Bradford total protein assay or by absorbance of light at 280 nm, however some reagents used during the purification process may interfere with the quantification. For example, imidazole (commonly used for purification of polyhistidine-tagged recombinant proteins) is an amino acid analogue and at low concentrations will interfere with the bicinchoninic acid (BCA) assay for total protein quantification. Impurities in low-grade imidazole will also absorb at 280 nm, resulting in an inaccurate reading of protein concentration from UV absorbance.

Another method to be considered is Surface Plasmon Resonance (SPR). SPR can detect binding of label free molecules on the surface of a chip. If the desired protein is an antibody, binding can be translated to directly to the activity of the protein. One can express the active concentration of the protein as the percent of the total protein. SPR can be a powerful method for quickly determining protein activity and overall yield. It is a powerful technology that requires an instrument to perform.

## Methods of protein purification

The methods used in protein purification can roughly be divided into analytical and preparative methods. The distinction is not exact, but the deciding factor is the amount of protein that can practically be purified with that method. Analytical methods aim to detect and identify a protein in a mixture, whereas preparative methods aim to produce large quantities of the protein for other purposes, such as structural biology or industrial use. In general, the preparative methods can be used in analytical applications, but not the other way around.

## Extraction

Depending on the source, the protein has to be brought into solution by breaking the tissue or cells containing it. There are several methods to achieve this: Repeated freezing and thawing, sonication, homogenization by high pressure, filtration (either via cellulose-based depth filters or cross-flow filtration[1]), or permeabilization by organic solvents. The method of choice depends on how fragile the protein is and how sturdy the cells are. After this extraction process soluble proteins will be in the solvent, and can be separated from cell membranes, DNA etc. by centrifugation. The extraction process also extracts proteases, which will start digesting the proteins in the solution. If the protein is sensitive to proteolysis, it is usually desirable to proceed quickly, and keep the extract cooled, to slow down proteolysis.

## Precipitation and differential solubilization

In bulk protein purification, a common first step to isolate proteins is precipitation with ammonium sulfate $(NH_4)_2SO_4$. This is performed by adding increasing amounts of ammonium sulfate and collecting the different fractions of precipitate protein. One advantage of this method is that it can be performed inexpensively with very large volumes.

The first proteins to be purified are water-soluble proteins. Purification of integral membrane proteins requires disruption of the cell membrane in order to isolate any one particular protein from others that are in the same membrane compartment. Sometimes a particular membrane fraction can be isolated first, such as isolating mitochondria from cells before purifying a protein located in a mitochondrial membrane. A detergent such as sodium dodecyl sulfate (SDS) can be used to dissolve cell membranes and keep membrane proteins in solution during purification; however, because SDS causes denaturation, milder detergents such as Triton X-100 or CHAPS can be used to retain the protein's native conformation during complete purification.

# Ultracentrifugation

*Main article: Ultracentrifuge*

Centrifugation is a process that uses centrifugal force to separate mixtures of particles of varying masses or densities suspended in a liquid. When a vessel (typically a tube or bottle) containing a mixture of proteins or other particulate matter, such as bacterial cells, is rotated at high speeds, the angular momentum yields an outward force to each particle that is proportional to its mass. The tendency of a given particle to move through the liquid because of this force is offset by the resistance the liquid exerts on the particle. The net effect of "spinning" the sample in a centrifuge is that massive, small, and dense particles move outward faster than less massive particles or particles with more "drag" in the liquid. When suspensions of particles are "spun" in a centrifuge, a "pellet" may form at the bottom of the vessel that is enriched for the most massive particles with low drag in the liquid. Non-compacted particles still remaining mostly in the liquid are called the "supernatant" and can be removed from the vessel to separate the supernatant from the pellet. The rate of centrifugation is specified by the angular acceleration applied to the sample, typically measured in comparison to the *g*. If samples are centrifuged long enough, the particles in the vessel will reach equilibrium wherein the particles accumulate specifically at a point in the vessel where their buoyant density is balanced with centrifugal force. Such an "equilibrium" centrifugation can allow extensive purification of a given particle.

Sucrose gradient centrifugation — a linear concentration gradient of sugar (typically sucrose, glycerol, or a silica based density gradient media, like Percoll) is generated in a tube such that the highest concentration is on the bottom and lowest on top. Percoll is a trademark owned by GE Healthcare companies. A protein sample is then layered on top of the gradient and spun at high speeds in an ultracentrifuge. This causes heavy macromolecules to migrate towards the bottom of the tube faster than lighter material. During centrifugation in the absence of sucrose, as particles move farther and farther from the center of rotation, they experience more and more centrifugal force (the further they move, the faster they move). The problem with this is that the useful separation range of within the vessel is restricted to a small observable window. Spinning a sample twice as long doesn't mean the particle of interest will go twice as far, in fact, it will go significantly further. However, when the proteins are moving through a sucrose gradient, they encounter liquid of increasing density and viscosity. A properly designed sucrose gradient will counteract the increasing centrifugal force so the particles move in close proportion to the time they have been in the centrifugal field. Samples separated by these gradients are referred to as "rate zonal" centrifugations. After separating the protein/particles, the gradient is then fractionated and collected.

# Chromatographic methods

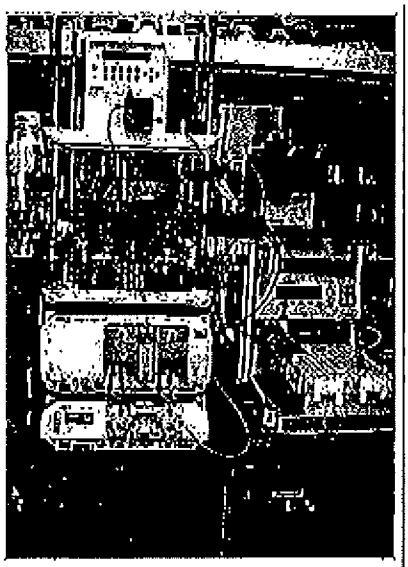
Usually a protein purification protocol contains one or more chromatographic steps. The basic procedure in chromatography is to flow the solution containing the protein through a column packed with various materials. Different proteins interact differently with the column material, and can thus be separated by the time required to pass the column, or the conditions required to elute the protein from the column. Usually proteins are detected as they are coming off the column by their absorbance at 280 nm. Many different chromatographic methods exist:

## Size exclusion chromatography

*Main article: Gel permeation chromatography*

Chromatography can be used to separate protein in solution or denaturing conditions by using porous gels. This technique is known as size exclusion chromatography. The principle is that smaller molecules have to traverse a larger volume in a porous matrix. Consequentially, proteins of a certain range in size will require a variable volume of eluent (solvent) before being collected at the other end of the column of gel.

In the context of protein purification, the eluant is usually pooled in different test tubes. All test tubes containing no measurable trace of the protein to purify are discarded. The remaining solution is thus made of the protein to purify and any other similarly-sized proteins.



Chromatographic equipment. Here set up for a size exclusion chromatography. The buffer is pumped through the column (right) by a computer controlled device.

## Separation based on charge or hydrophobicity

Hydrophobic Interaction Chromatography

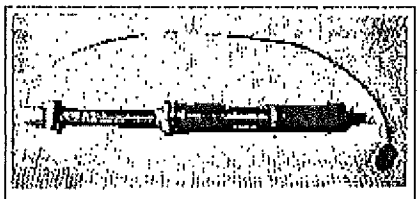
## Ion exchange chromatography

*Main article: Ion exchange chromatography*

Ion exchange chromatography separates compounds according to the nature and degree of their ionic charge. The column to be used is selected according to its type and strength of charge. Anion exchange resins have a positive charge and are used to retain and separate negatively charged compounds, while cation exchange resins have a negative charge and are used to separate positively charged molecules.

Before the separation begins a buffer is pumped through the column to equilibrate the opposing charged ions. Upon injection of the sample, solute molecules will exchange with the buffer ions as each competes for the binding sites on the resin. The length of retention for each solute depends upon the strength of its charge. The most weakly charged compounds will elute first, followed by those with successively stronger charges. Because of the nature of the separating mechanism, pH, buffer type, buffer concentration, and temperature all play important roles in controlling the separation.

Ion exchange chromatography is a very powerful tool for use in protein purification and is frequently used in both analytical and preparative separations.



Nickel-affinity column. The resin is blue since it has bound nickel.

## Affinity chromatography

*Main article: Affinity chromatography*

Affinity Chromatography is a separation technique based upon molecular conformation, which frequently utilizes application specific resins. These resins have ligands attached to their surfaces which are specific for the compounds to be separated. Most frequently, these ligands function in a fashion similar to that of antibody-antigen interactions. This "lock and key" fit between the ligand and its target compound makes it highly specific, frequently generating a single peak, while all else in the sample is unretained.

Many membrane proteins are glycoproteins and can be purified by lectin affinity chromatography. Detergent-solubilized proteins can be allowed to bind to a chromatography resin that has been modified to have a covalently attached lectin. Proteins that do not bind to the lectin are washed away and then specifically bound glycoproteins can be eluted by adding a high concentration of a sugar that competes with the bound glycoproteins at the lectin binding site. Some lectins have high affinity binding to oligosaccharides of glycoproteins that is hard to compete with sugars, and bound glycoproteins need to be released by denaturing the lectin.

### Metal binding

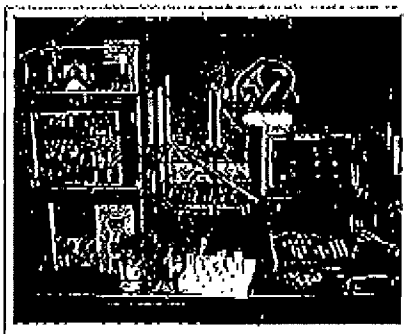
*Main article: Polyhistidine-tag*

A common technique involves engineering a sequence of 6 to 8 histidines into the N- or C-terminal of the protein. The polyhistidine binds strongly to divalent metal ions such as nickel and cobalt. The protein can be passed through a column containing immobilized nickel ions, which binds the polyhistidine tag. All untagged proteins pass through the column. The protein can be eluted with imidazole, which competes with the polyhistidine tag for binding to the column, or by a decrease in pH (typically to 4.5), which decreases the affinity of the tag for the resin. While this procedure is generally used for the purification of recombinant proteins with an engineered affinity tag (such as a 6xHis tag or Clontech's HAT tag), it can also be used for natural proteins with an inherent affinity for divalent cations.

### Immunoaffinity chromatography

*Main article: Immunoaffinity chromatography*

Immunoaffinity chromatography uses the specific binding of an antibody to the target protein to selectively purify the protein. The procedure involves immobilizing an antibody to a column material, which then selectively binds the protein, while everything else flows through. The protein can be eluted by changing the pH or the salinity. Because this method does not involve engineering in a tag, it can be used for proteins from natural sources.[2]



A HPLC. From left to right: A pumping device generating a gradient of two different solvents, a steel enforced column and an apparatus for measuring the absorbance.

#### Purification of a tagged protein

Adding a tag to the protein such as RuBPS gives the protein a binding affinity it would not otherwise have. Usually the recombinant protein is the only protein in the mixture with this affinity, which aids in separation. The most common tag is the Histidine-tag (His-tag), that has affinity towards nickel or cobalt ions. Thus by immobilizing nickel or cobalt ions on a resin, an affinity support that specifically binds to histidine-tagged proteins can be created. Since the protein is the only component with a His-tag, all other proteins will pass through the column, and leave the His-tagged protein bound to the resin. The protein is released from the column in a process called elution, which in this case involves adding imidazole, to compete with the His-tags for nickel binding, as it has a ring structure similar to histidine. The protein of interest is now the major protein component in the eluted mixture, and can easily be separated from any minor unwanted contaminants by a second step of purification, such as size exclusion chromatography or RP-HPLC.

Another way to tag proteins is to engineer an antigen peptide tag onto the protein, and then purify the protein on a column or by incubating with a loose resin that is coated with an immobilized antibody. This particular procedure is known as immunoprecipitation. Immunoprecipitation is quite capable of generating an extremely specific interaction which usually results in binding only the desired protein. The purified tagged proteins can then easily be separated from the other proteins in solution and later eluted back into clean solution.

When the tags are not needed anymore, they can be cleaved off by a protease. This often involves engineering a protease cleavage site between the tag and the protein.

### HPLC

*Main article: High performance liquid chromatography*

High performance liquid chromatography or high pressure liquid chromatography is a form of chromatography applying high pressure to drive the solutes through the column faster. This means that the diffusion is limited and the resolution is improved. The most common form is "reversed phase" hplc, where the column material is hydrophobic. The proteins are eluted by a gradient of increasing amounts of an organic solvent, such as acetonitrile. The proteins elute according to their hydrophobicity. After purification by HPLC the protein is in a solution that only contains volatile compounds, and can easily be lyophilized.[3] HPLC purification frequently results in denaturation of the purified proteins and is thus not applicable to proteins that do not spontaneously refold.

## Concentration of the purified protein

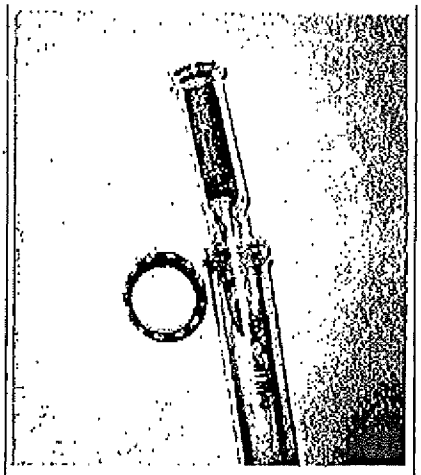
At the end of a protein purification, the protein often has to be concentrated. Different methods exist.

### Lyophilization

If the solution doesn't contain any other soluble component than the protein in question the protein can be lyophilized (dried). This is commonly done after an HPLC run. This simply removes all volatile component leaving the proteins behind.

### Ultrafiltration

Ultrafiltration concentrates a protein solution using selective permeable membranes. The function of the membrane is to



A selectively permeable membrane can be mounted in a centrifuge tube. The buffer is forced through the membrane by centrifugation, leaving the protein in the upper chamber.

ɔt the water and small molecules pass through ( e retaining the protein. The solution is forced against the membrane by mechanical pump or gas pressure or centrifugation.

## Analytical

### Denaturing-Condition Electrophoresis

Gel electrophoresis is a common laboratory technique that can be used both as preparative and analytical method. The principle of electrophoresis relies on the movement of a charged ion in an electric field. In practice, the proteins are denatured in a solution containing a detergent (SDS). In these conditions, the proteins are unfolded and coated with negatively charged detergent molecules. The proteins in SDS-PAGE are separated on the sole basis of their size.

In analytical methods, the protein migrate as bands based on size. Each band can be detected using stains such as Coomassie blue dye or silver stain. Preparative methods to purify large amounts of protein, require the extraction of the protein from the electrophoretic gel. This extraction may involve excision of the gel containing a band, or eluting the band directly off the gel as it runs off the end of the gel.

In the context of a purification strategy, denaturing condition electrophoresis provides an improved resolution over size exclusion chromatography, but does not scale to large quantity of proteins in a sample as well as the late chromatography columns.

### Non-Denaturing-Condition Electrophoresis

An important non-denaturing electrophoretic procedure for isolating bioactive metalloproteins in complex protein mixtures is termed 'quantitative native continuous polyacrylamide gel electrophoresis (QPNC-PAGE).

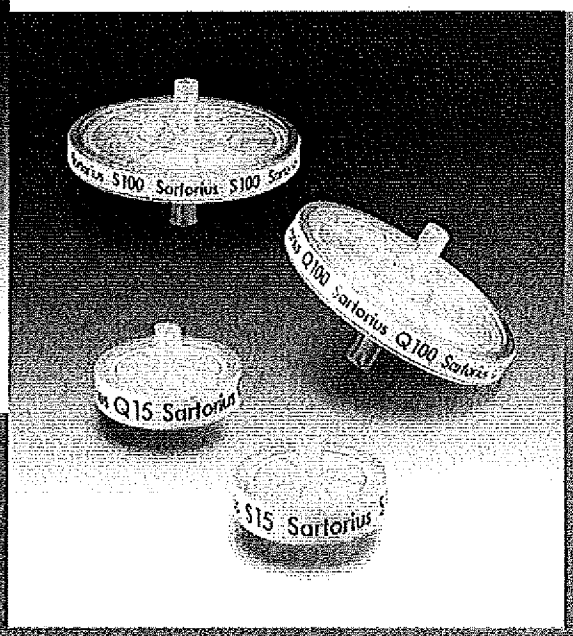
## References


1. ^ Millipore Technical Library: Protein Concentration and Diafiltration by Tangential Flow Filtration (http://www.millipore.com/publications.nsf/docs/tb032)
2. ^ Immunoaffinity chromatography of enzymes (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=pubmed&dopt=Abstract&list_uids=1368167&query_hl=6&itool=pubmed_DocSum) Ehle H, Horn A. Bioseparation. 1990;1(2):97-110.
3. ^ High-performance liquid chromatography of biopolymers (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=pubmed&dopt=Abstract&list_uids=6353575&query_hl=10&itool=pubmed_DocSum) Regnier FE Science. 1983 Oct 21;222(4621):245-52


## External links

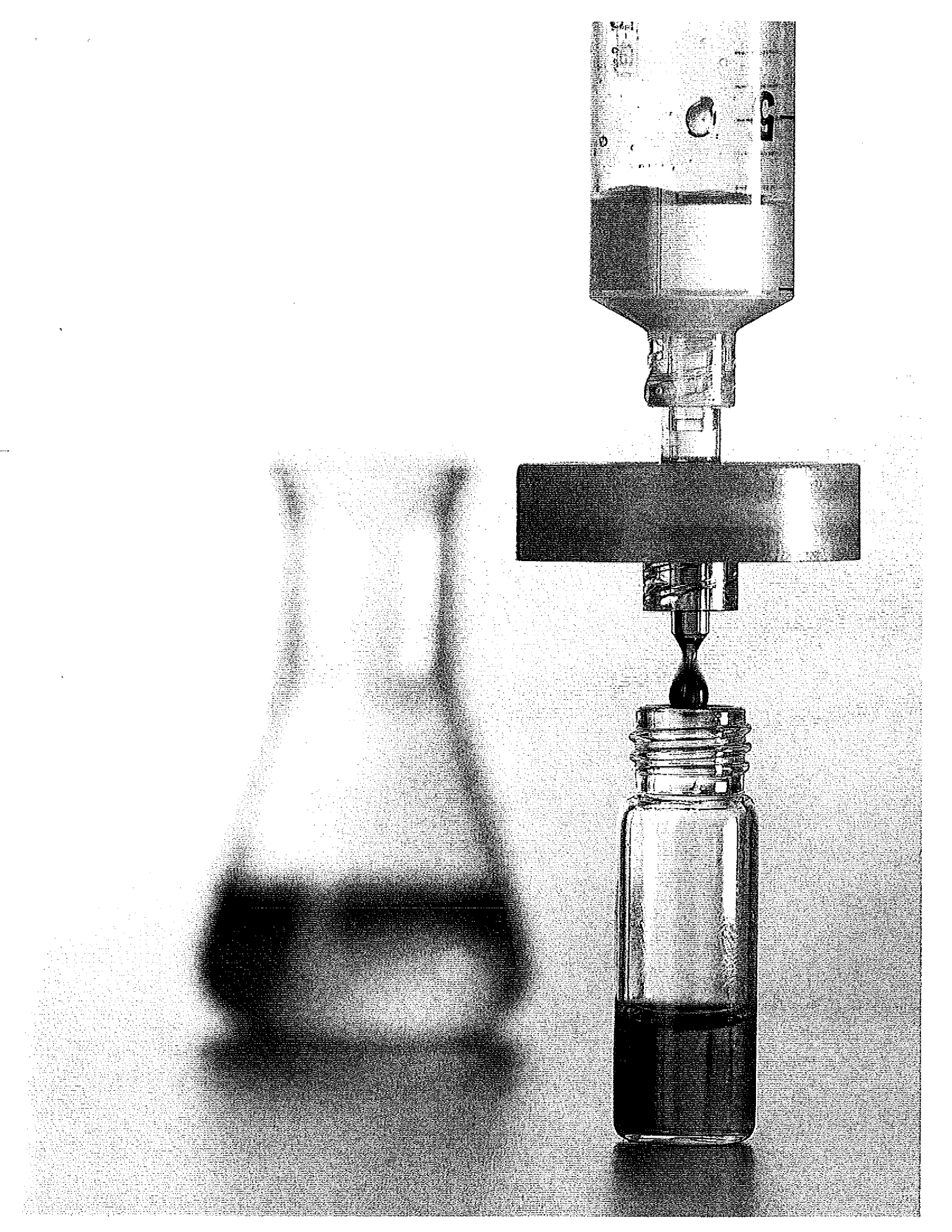
- Protein purification in one day (http://proteincrystallography.org/protein-purification/)
- Protein purification facility (http://wolfson.huji.ac.il/purification/)
- Slope Spectroscopy (http://www.solovpe.com/)

sartorius




# Sartobind®

## Membrane Adsorbers for Rapid Purification of Proteins



# Sartobind – the pace maker in Membrane Adsorber technology

**Unique microporous structure**
Sartobind Membrane Adsorbers display a microporous structure with a pore size of 0.45 or > 3 µm. That's orders of magnitudes larger than conventional chromatographic gel matrices. Molecules are transported by convective flow to ligands.

**Characteristics of Membrane Adsorbers (MA)**
- Ready-to-use units
- Simple handling with a syringe or with a pump
- Pore sizes > 3 and 0.45 µm
- Negligible diffusion limitation
- Low bed heights between 0.3 and 4 mm, Sartobind MultiSep process modules up to 16 mm
- Scalable to process dimension with Sartobind SingleSep disposable capsules and Sartobind MultiSep reusable modules
- Flow starts already at 10 kPa
- Robust high performance separations
- No bed cracking, channeling, air entrapment
- 1 m² membrane ≡ 100 m² internal surface
- Flow rate of ion exchange membranes > 80 ml/min 100 kPa (linear flow rate: > 4,800 cm/h)
- Chemistries: strong and weak ion exchange, coupling, affinity and metal chelate ligands

**Low unspecific adsorption**
The basis for all Sartobind membranes is a stabilized reinforced cellulose. It is made from regenerated cellulose and during the production to Sartobind it runs through a number of stabilization and grafting steps until a chromatographic matrix is formed on the cellulose backbone. In principle any ligands known from conventional chromatography can be bound covalently on the matrix.

**Speed up 100 times**
In a simple experiment using a Sartobind ion exchange unit with 5 cm² membrane area and a Luer Lock syringe you can achieve a flow rate of about 10 ml per 0.5 second by hand which corresponds to a linear flow rate of more than 14,000 cm/h. Even under these conditions you'll attain complete capture of your protein. Just try it (sample: cytochrome c, buffer: 10 mM sodium phosphate pH 7.0, unit: Sartobind S 5). In production scale (more than 10,000 liters), a typical speed up factor is about 25 measured in direct comparison to conventional column technology (reference: Walter, J. K. in: Bioseparation and Bioprocessing, Strategies and Considerations for Advanced Economy in Downstream Processing of Biopharmaceutical Proteins. G. Subramanian (ed.) Wiley VCH, Vol. II p. 447–460 (1998). Flow rate does not affect break through performance (see fig. 2).

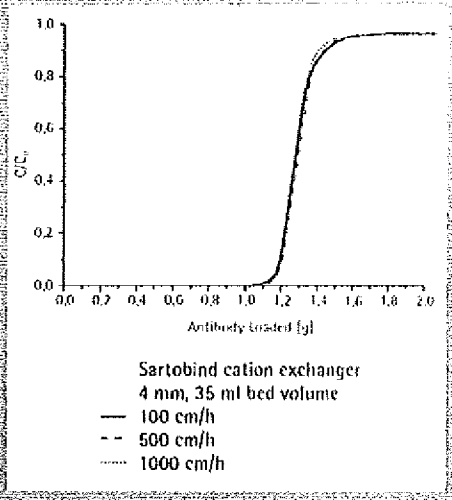



Fig. 2: Break through performance of a Sartobind MultiSep 35 ml cation exchange module at monoclonal antibody purification with various flow rates. For other published data see also: H. L. Knudsen et al., Genentech Inc., J. Chromatogr. A 907 (2001) 145–154.

**Sartobind membrane types**
- Sartobind S, Q, C and D ion exchange
- Sartobind IDA (iminodiacetic acid) metal chelate
- Sartobind aldehyde-activated
- Sartobind epoxy-activated
- Sartobind Protein A (recombinant)
- Other ligands on request

**Sartobind applications**
Purification and Concentration
- Proteins, viruses, viral particles, monoclonal antibodies, oligonucleotides

Contaminant removal
- DNA, endotoxins, viruses, host cell, proteins

◀ Fig. 1: Cytochrome c is eluted from a Sartobind S 75 Membrane Adsorber

### Constant capacity

The robustness of Sartobind Membrane Adsorbers in ion exchange and affinity chromatography has been tested in consecutive runs of 1000 or 100 chromatographic cycles, respectively (fig. 3 and 4). Sartobind can be reused many times, and affinity membranes maintain their binding capacity over many cycles reflecting the chemical stability and low "bleeding" of ligand.

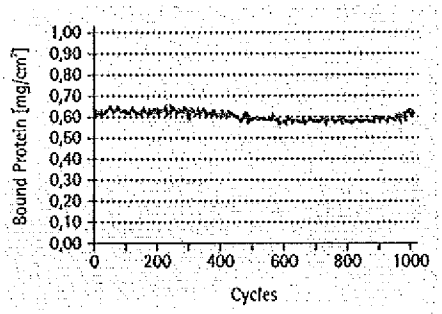



Fig. 3: Purification cycles of bovine serum albumin on Sartobind Q 75 strong anion exchanger repeated 1000 times. Flow rate: 120 cm/h, cycle time: 10 min, equilibration buffer: 20 mM phosphate buffer pH 7.0, sample: 5 ml bovine serum diluted 1:20 with equilibratioin buffer, elution buffer: 20 mM phosphate buffer pH 7.0 + 1 N NaCl, regeneration after each 100 cycles with 1 N NaOH

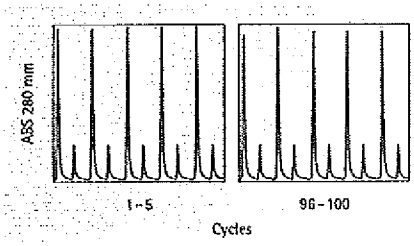



Fig. 4: Purification cycles of IgG on Sartobind recombinant protein A 3 (3 layer unit) repeated 100 times. Elution pattern of cycles 1–5 (left) and 96–100. Flow rate: 193 cm/h , cycle time: 7 min, equilibration and washing buffer: PBS 1 x, sample: human plasma, elution buffer: glycine 0.1 M, pH 2.3. The first peak of each cycle is the flow through of unbound proteins, mainly HSA (human serum albumin), second peak represents the eluted IgG fraction.

### Laboratory units: Sartobind MA

You may use Sartobind Membrane Adsorbers for any ion exchange or affinity chromatography which require high speed and simple operation. Four sizes of laboratory units can be chosen. Sartobind MA 5 (1 membrane layer) are for principle tests and should be disposed after use. If you have to work with larger mg quantities, please choose Sartobind MA 15 (3 layers), MA 75 (15 layers) or MA 100 (5 layers). These may be reused hundreds of times.

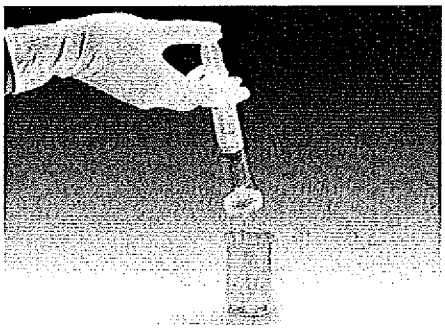


Fig. 5: Sartobind MAs may be used by hand or with a chromatographic system via Luer Lock adapters.

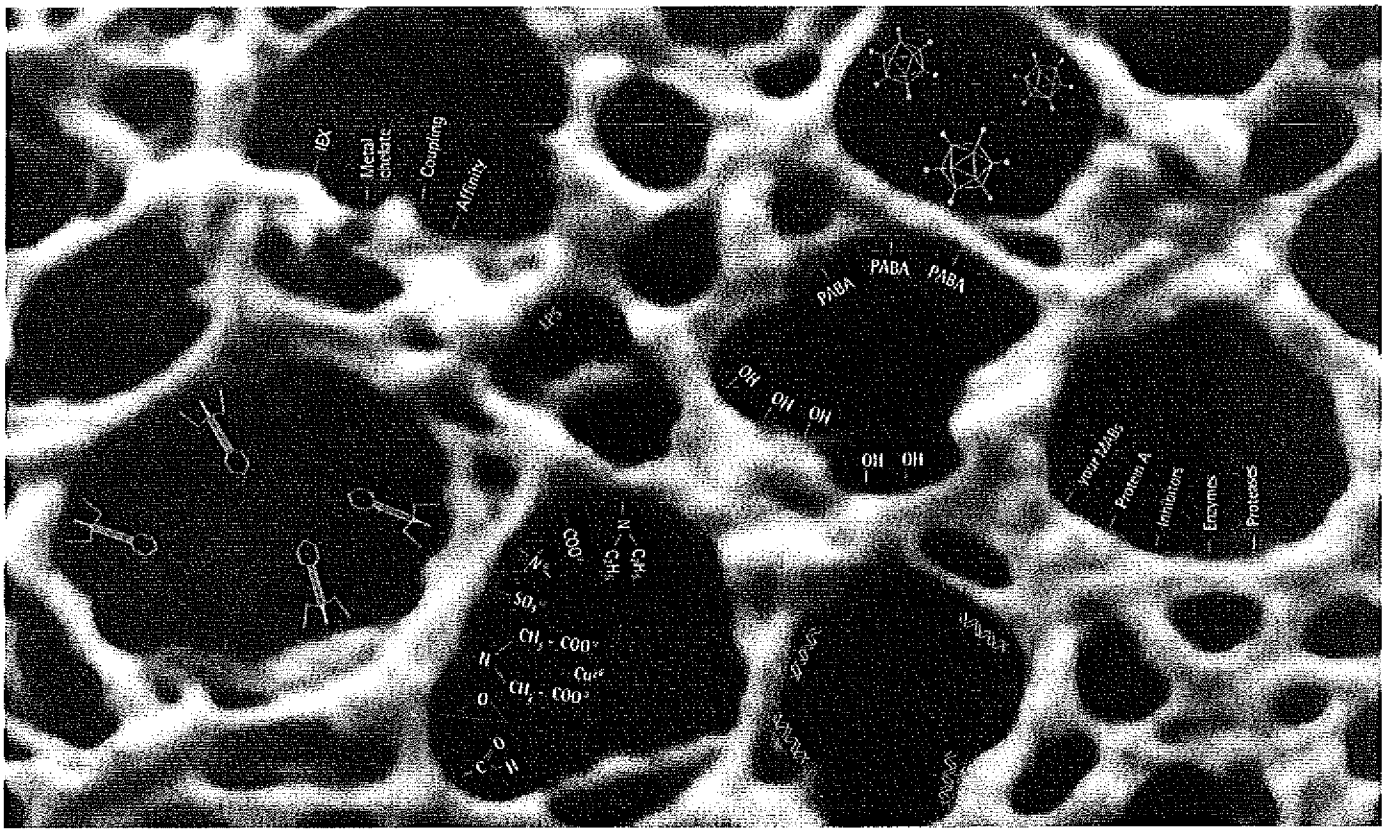



Fig. 6: There are no restrictions regarding ligands and applications.

# Formats from lab to process

**Centrifugal format**
If you don't need flow control but you have to screen a large number of proteins in parallel, please ask for the Vivapure® brochure.

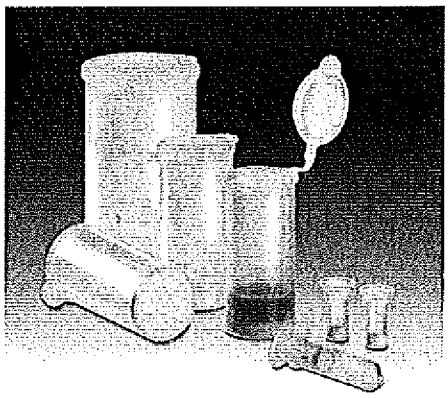


Fig. 7: Vivapure centrifugal units

**Sartobind SingleSep, single use capsules**
Our well known capsules have been equipped with Sartobind to supply a simple format for contaminant removal in production.
For technical data and description ask for the SingleSep information.

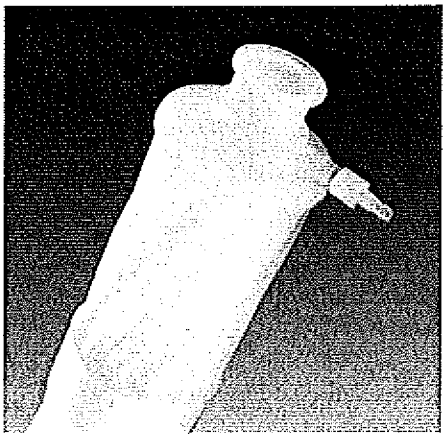


Fig. 9: Sartobind SingleSep 10" capsule

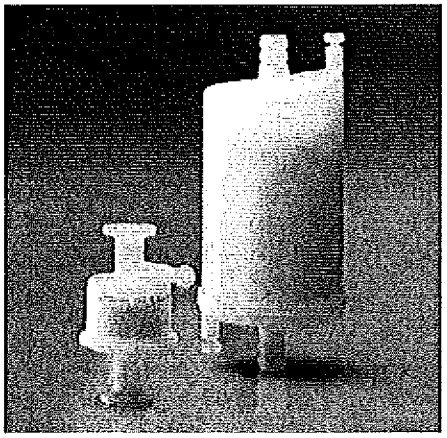


Fig. 11: Sartobind SingleSep mini and 5" capsules

**Production formats**
**Sartobind MultiSep, reusable modules**
The MultiSep modules represent a unique modular system for any chromatographic application with Membrane Adsorbers. Modules are available in 15 sizes and with protein capacities between 1 and 60 g (IEX). Module can be run in series and parallel for further scale-up. For technical data and description ask for the MultiSep information package.

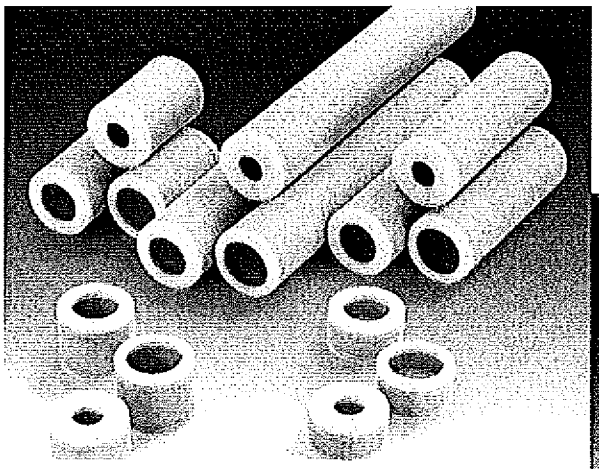


Fig. 8: Sartobind MultiSep modules

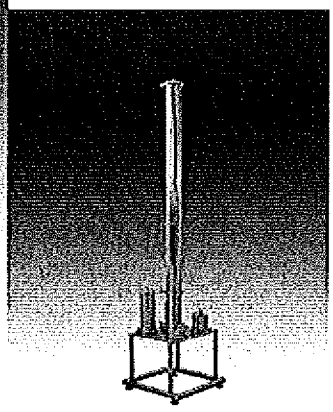


Fig. 10: Sartobind MultiSep for production scale

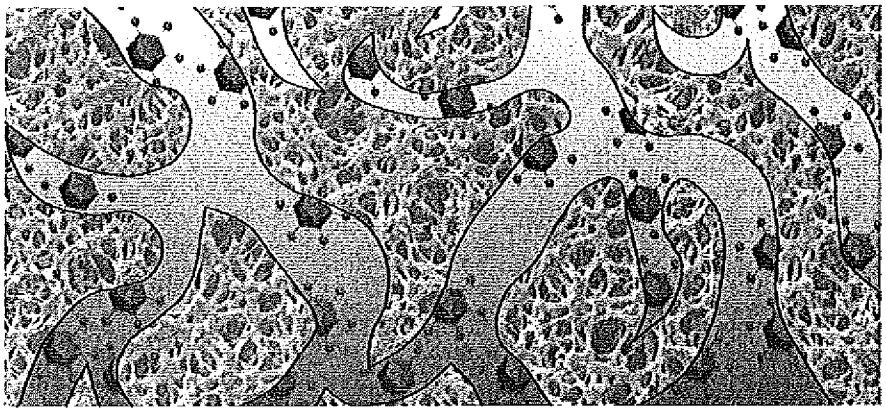
**Starting with Sartobind MA for further scale-up**
The Sartobind MA 75 format with 4 mm bed height is available in all membrane types. It can be scaled straightly to the production format MultiSep containing the same bed height.

Fig. 12: Sartobind MA units for laboratory applications

**Chemical compatibility**
The material of Sartobind MA 5 housing is MBS (methyl methacrylate butadiene styrene) copolymer and is intended for single use only. The housing of Sartobind MA 15, 75 and 100 is polysulfone which is stable to many standard solvents applied in chromatography. Sartobind ion exchange membranes are compatible with alcohols such as ethanol, isopropanol, glycerol, and denaturing solvents such as 8 M urea and 8 M guanidine HCl. They resist strong acids and alkaline solutions such as 1 M hydrochloric and sulfuric acid, 1 N sodium hydroxide acid and 1 N sodium hydroxide.

# A standard for virus purification

**Unique for large proteins, DNA and viruses**
Sartobind membranes are unique for purification of large proteins and virus particles. Conventional gel beads possess 95% of the binding capacity within the bead and large particles cannot enter their small pores. In contrast Sartobind membranes display huge capacities for large molecules. E.g. for DNA 10 times more dynamic capacity than conventional beads (reference: Sartobind Application Note DNA removal) and for virus purifications gels cannot compete at all. Sartobind membranes have been successfully applied for the purification of alphaherpesvirus (Sartobind S 15), adenovirus and adenoassociated virus (Sartobind Q 15).



**Certification**
The products must meet all Sartorius standards for traceability and production. Sartorius manufactures certified Sartobind MultiSep, SingleSep, Sartobind MA 5, 15, 75 and 100 as well as all Sartobind membranes in a controlled environment. Sartobind SingleSep and MultiSep have met the specifications for current USP plastic class VI tests.

**Quality control**
Membranes are tested for protein binding capacity, thickness, evenness, smoothness and flow rate. Sartobind MA laboratory units are tested for protein binding capacity and flow rate. All capsules and modules are tested for small ion capacity and flow rate.

**FDA approved**
Sartobind is the first Membrane Adsorber applied in approved pharmaceutical production (approval of CAMPATH-1H® by Food and Drug Administration, March 2001).

**Validated technology**
Validation and extractables guides are available containing detailed test descriptions and extractables analysis using GC-MS, HPLC and FT-IR from aqueous, ethanolic and sodium hydroxide extraction samples at different temperatures.

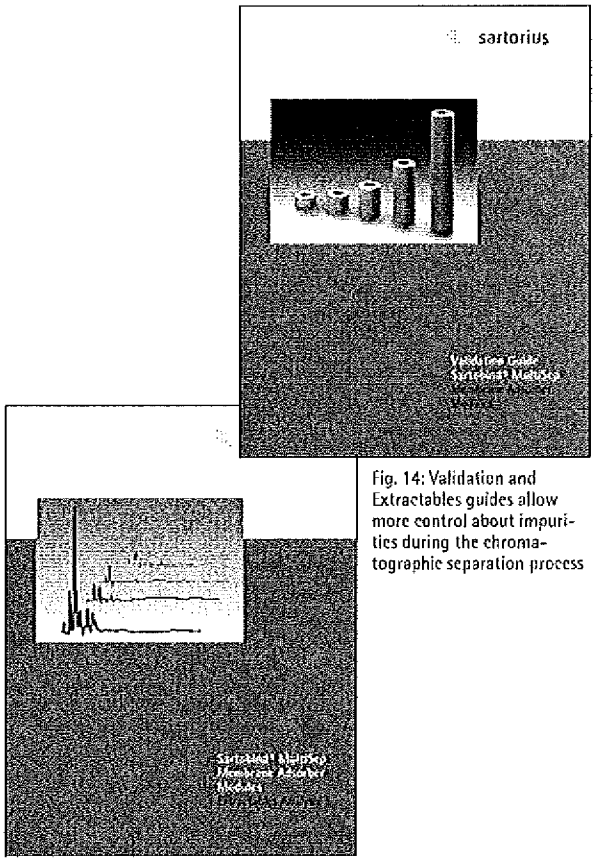
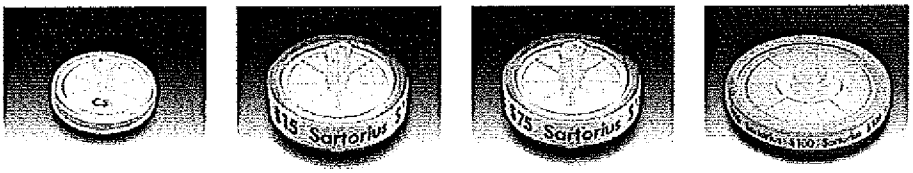



Fig. 14: Validation and Extractables guides allow more control about impurities during the chromatographic separation process

## Technical Data

Sartobind MA




| | MA 5 | MA 15 | MA 75 | MA 100 |
|---|---|---|---|---|
| Membrane material | Stabilized reinforced cellulose | | | |
| Application for | Principle tests only, single use | Purification, reusable | Purification, down-scale for production, reusable | Purification, reusable |
| Adsorption area [cm²] | 5 | 15 | 75 | 100 |
| Number of layers | 1 | 3 | 15 | 5 |
| Bed height [mm] | 0.275 | 0.8 | 4.0 | 1.4 |
| Bed volume* [ml] | 0.14 | 0.41 | 2.1 | 2.8 |
| Menbrane diameter [mm] | 25 | 25 | 25 | 50 |
| Housing material | MBS copolymer | Polysulfone | Polysulfone | Polysulfone |
| Inlet connector | Female Luer Lock | Female Luer Lock | Female Luer Lock | Female Luer Lock |
| Outlet connector | Male Luer Lock | Male Luer Lock | Male Luer Lock | Male Luer Lock |
| Minimum static protein binding capacity [mg/unit] | 4 / S 5<br>4 / Q 5<br>3 / C 5<br>3 / D 5 | 12 / S 15<br>12 / Q 15<br>9 / C 15<br>9 / D 15 | 60 / S 75<br>60 / Q 75<br>45 / C 75<br>45 / D 75<br>2.2 / Epoxy 75<br>6 / Protein A 75<br>7.5 / IDA 75 | 80 / S 100<br>80 / Q 100<br>60 / C 100<br>60 / D 100 |
| Flow rate** at 0.1 MPa (1 bar \| 14.5 psi) [ml/min] | >150 | >50 | >20 | >75 |
| Dead volume [ml] | 0.8 | 1.0 | 1.3 | 4.2 |
| Maximum pressure [MPa] | 0.4 | 0.6 | 0.6 | 0.6 |
| pH stability of housing | – | 2–13 | 2–13 | 2–13 |
| Storage before use at | Room temperature | Room temperature | Room temperature<br>Protein A 75 at +4°C | Room temperature |

* 1 ml membrane volume is equal to 36.4 cm² membrane area
** ion exchange

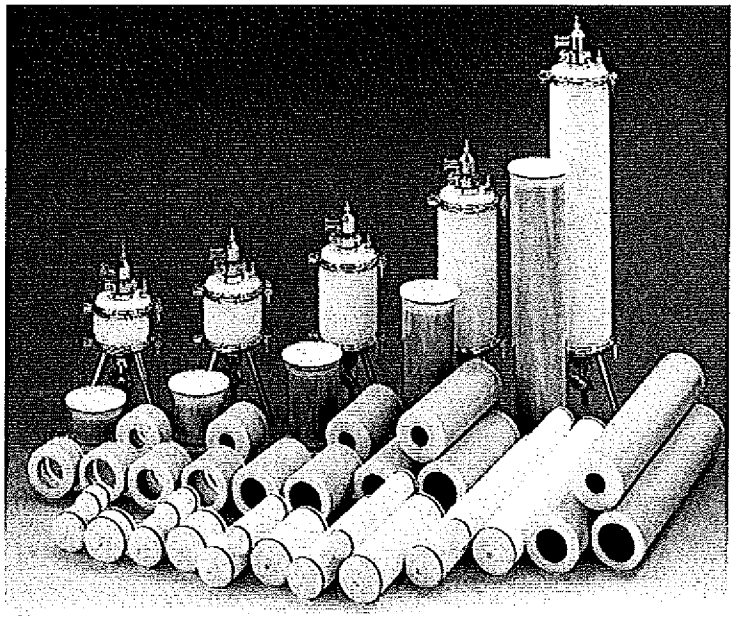


Fig. 15: Sartobind MultiSep products: housings, storage containers, modules and cores

## Product and ordering information

| Sartobind Membrane | Description | Pore size (µm) | Capacity (µg/cm )* | Sartobind MA 5 | Sartobind MA 15 | Sartobind MA 75 | Sartobind MA 100 | Sartobind SingleSep | Sartobind MultiSep |
|---|---|---|---|---|---|---|---|---|---|
| S | Strong acidic cation exchanger sulfonic acid | >3 | 800 | • | • | • | • | • | • |
| Q | Strong basic anion exchanger quaternary ammonium | >3 | 800 | • | • | • | • | • | • |
| C | Weak acidic cation exchanger carboxylic acid | >3 | 600 | • | • | • | • | | • |
| D | Weak basic anion exchanger diethylamine | >3 | 600 | • | • | • | • | | • |
| IDA | Iminodiacetic acid metal chelate | >3 | 100 | | | • | | | • |
| Epoxy | Epoxy-activated membrane | 0.45 | 30 | | | • | | | • |
| Protein A | Recombinant protein A affinity | 0.45 | 80 | | | • | | | • |
| Aldehyde membrane | Aldehyde-activated | 0.45 | 28 | | | • | | | • |

* Minimum static capacity for standard or target protein, • available

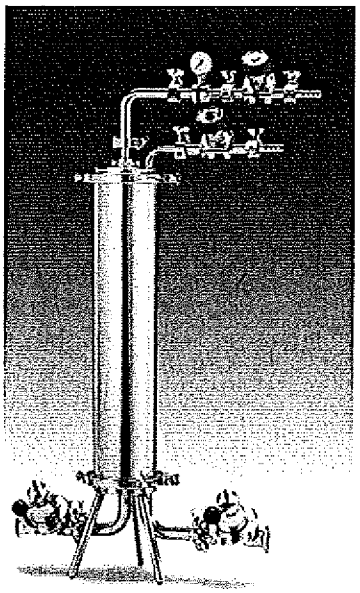


Fig. 16: MultiSep T-style housing

| Order No. | Description | Quantity |
|---|---|---|
| S5F | Sartobind S 5 strong cation exchanger | 15 |
| Q5F | Sartobind Q 5 strong anion exchanger | 15 |
| C5F | Sartobind C 5 weak cation exchanger | 15 |
| D5F | Sartobind D 5 weak anion exchanger | 15 |
| S15X | Sartobind S 15 strong cation exchanger | 2 |
| Q15X | Sartobind Q 15 strong anion exchanger | 2 |
| C15X | Sartobind C 15 weak cation exchanger | 2 |
| D15X | Sartobind D 15 weak anion exchanger | 2 |
| Q75X | Sartobind Q 75, strong anion exchanger | 2 |
| S75X | Sartobind S 75, strong cation exchanger | 2 |
| C75X | Sartobind C 75, weak cation exchanger | 2 |
| D75X | Sartobind D 75, weak anion exchanger | 2 |
| S100X | Sartobind S 100 strong cation exchanger | 1 |
| Q100X | Sartobind Q 100 strong anion exchanger | 1 |
| C100X | Sartobind S 100 weak cation exchanger | 1 |
| D100X | Sartobind S 100 weak anion exchanger | 1 |

| Order No. | Description | Quantity |
|---|---|---|
| 93PR-A06DB-12--V | Sartobind Protein A 75, recombinant protein A | 2 |
| 93IDA-42DB-12--V | Sartobind IDA 75, iminodiacetic acid | 2 |
| 93EPOX06DB-12--V | Sartobind Epoxy 75, epoxy activated | 2 |
| 17002---140 | Luer Lock adapters black Tefzel to connect MA 5, 15, 75 and 100 to a liquid chromatography system | 2 |

**Related products for production**

Sartobind SingleSep capsules
Sartobind MultiSep modules
Sartobind MultiSep cores
Sartobind MultiSep housings plastic
Sartobind MultiSep housings stainless steel
Sartobind MultiSep plastic containers

Fig. 17: Sartobind MA units can be connected in series ▶

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Vivapure® is a trademark of Vivascience AG (Sartorius group)
CAMPATH® is a trademark of Millennium Pharmaceuticals Inc.


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Publication No.: SL-1513-e03081
Order No.: 85030-518-92

Nephrol Dial Transplant (1996) 11: 1113–1120

Nephrology
Dialysis
Transplantation

*Original Article*

# Effect of electric charge on the transperitoneal transport of plasma proteins during CAPD

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[1]Academic Medical Center, University of Amsterdam, Department of Internal Medicine, Renal Unit, Amsterdam; [2]Foundation for Home-Dialysis Midden-West Nederland; [3]Sint Radboud Hospital, University of Nijmegen, Department of Pediatric Nephrology, Nijmegen, The Netherlands

**Abstract**

**Background.** Controversy exists as to whether electric charges of plasma proteins influence their transport across the peritoneal membrane during CAPD. Fixed negative charges in the peritoneal membrane are diminished during peritonitis in rats.

**Methods.** Peritoneal clearances of 10 proteins and their isoforms were assessed in CAPD patients. The most neutral proteins were used to establish the relationship between peritoneal clearance and molecular weight. The observed protein clearances were compared with the predicted clearances based on molecular weight. Clearances of proteins with different charge but identical size were compared. Stable patients and peritonitis patients were compared.

**Results.** Only the peritoneal clearance of lipase, LDH 4/5 and IgG3 were significantly different from the predicted values ($P \leqslant 0.05$). The peritoneal clearance of slightly anionic $\beta_2$ microglobulin (1072 µl/min) and cationic lysozyme (572 µl/min) showed no evidence for charge selectivity; neither did the peritoneal clearance of slightly anionic transferrin (86 µl/min) and highly anionic albumin (99 µl/min). The peritoneal clearance of IgG1, IgG2 and IgG4 were identical (32, 31 and 31 µl/min), despite their different charge. The peritoneal clearance of cationic LDH 4/5 was 137 µl/min and higher than the peritoneal clearance of neutral LDH 3 (97 µl/min, $P=0.01$) and anionic LDH 2 (65 µl/min, $P=0.01$) and LDH 1 (59 µl/min, $P=0.02$). These results suggested charge selectivity; however in five additional patients during peritonitis the peritoneal clearance of LDH 4/5 increased to 10 times the peritoneal clearance of LDH 1. Local LDH isoenzyme release from the cells present in the dialysate was shown to be responsible in stable and peritonitis patients. Likewise, the higher peritoneal clearance of neutral pancreatic amylase (234 µl/min) compared to anionic salivary amylase (142 µl/min, $P=0.03$) could probably be attributed to local release of the former from the pancreas, as the peritoneal clearance of lipase (highly anionic) was higher than predicted and the difference remained during peritonitis.

**Conclusions.** The peritoneal membrane constitutes a size- but probably not a charge-selective barrier for the transport of macromolecules between blood and dialysate during stable CAPD.

**Key words:** CAPD; charge selectivity; LDH; peritoneal membrane; peritonitis; proteins

## Introduction

Plasma proteins are present in the peritoneal effluent of CAPD (continuous ambulatory peritoneal dialysis) patients. This leads to a peritoneal protein loss that averages 5–10 g per day in stable patients [1,2], but can increase to 20 g during peritonitis [3]. Although this protein loss does not lead to overt depletion of specific proteins, adequate amino acid intake is required to avoid protein malnutrition. For the transport from the circulation to the peritoneal cavity, plasma proteins have to pass the peritoneal membrane. This transperitoneal protein transport was previously shown to be size selective [4]. Fixed negative charges have been reported to be present in the peritoneal membrane of animals not treated with peritoneal dialysis [5,6]. These negative charges were reduced in rats during septic peritonitis [7,8]. However, their importance in the transperitoneal transport of macromolecules is not established. Previously we found no indication that the electric charge of a macromolecule is a determinant of its transperitoneal transport rate [9,10]. Other studies indicated that negatively charged macromolecules are retarded in their passage across the peritoneal membrane [11,12], but the opposite has also been found using neutral and charged dextrans [13].

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An attractive way to study the effect of electric charge in CAPD patients is the comparison of the clearances of proteins with identical or almost identical size and shape, but with different isoelectric points. The aim of the present study was therefore to determine the contribution of the electric charge of plasma proteins to their transperitoneal transport in stable CAPD patients and during peritonitis. This was done by comparing the peritoneal clearances of $\beta_2$ microglobulin and lysozyme, of pancreas- and saliva-amylase, of albumin and transferrin, of lactate dehydrogenase (LDH) isoenzymes, and of immunoglobulin G (IgG) subclasses. The clearance of lipase was used to assess a possible contribution of leakage of pancreatic enzymes from the pancreas.

## Subjects and methods

### *Patients*

Nine stable patients (3 females and 6 males; aged 22–69 years, median 50 years) were studied. They were treated with CAPD for 3–71 months (median 50 months). All patients were free of peritonitis during the study and in the 4 preceding weeks. In addition five patients were studied on the first or second day of an episode of bacterial peritonitis. On the basis of the first results in these patients, lysates were made from the cells present in the peritoneal effluent of five patients during peritonitis and five additional stable patients (night dwell).

### *Study protocol*

The nine stable patients were studied during a 6-h dwell with commercially available dialysate (Dianeal$^R$ 2.27%, Baxter, The Netherlands), to which dextran 70 (Macrodex$^R$) 1 g/l was added. Prior to the test, 20 ml of dextran 1 (Promiten$^R$, NPBI, Emmercompascuum, The Netherlands) 150 mg/ml was given intravenously to prevent possible anaphylaxis to dextran 70 [14]. The peritoneal cavity was rinsed with dialysate before the test bag was instilled; after 6 h, the test solution was drained by gravity. Immediately following drainage, the peritoneal cavity was rinsed with a fresh dialysate solution (Dianeal$^R$ 2.27%), from which samples were drawn for the calculation of the residual volume.

### *Sample collection and analysis*

An EDTA blood sample was obtained during drainage of the effluent and centrifuged immediately. A dialysate sample was centrifuged; the supernatant was concentrated 7- to 10-fold by means of positive pressure ultrafiltration using a 50-ml cell and a YM-10 membrane (Amicon Corp., Danvers, Massachusetts, USA). The molecular cutoff of this membrane is 10 kDa. All protein concentrations that were measured in the concentrated dialysate (total and pancreatic amylase, lipase, LDH isoenzymes, IgG, IgG subclasses, $\alpha_2$ macroglobulin) were corrected by dividing them by a concentration factor defined as: $[\text{albumin}]_{\text{concentrate}}/[\text{albumin}]_{\text{original sample}}$. $\beta_2$ Microglobulin, amylase, albumin, IgG, and $\alpha_2$ microglobulin were measured in the ultrafiltrate but were not detectable.

All measurements were done in freshly obtained plasma and dialysate within 1 week; samples were stored at 4°C until determination.

Concentrations of $\alpha_1$ microglobulin, albumin, transferrin, IgG, and $\alpha_2$ macroglobulin were measured by a nephelometric method (BN 100, Behring). Antisera were obtained from Behring (Behringwerke, Marburg, Germany) except for anti-IgG (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands). $\beta_2$ Microglobulin was measured using a test kit for an IMX system (Abbott Laboratories, Illinois, USA). Lysozyme concentration was measured in an ELISA using a rabbit anti-lysozyme antiserum obtained from DAKO A/S, Glostrup, Denmark. Pancreatic and total α-amylase were measured spectrophotometrically using a commercial kit (Boehringer Mannheim, Mannheim, Germany); salivary amylase activity was obtained by subtracting pancreatic from total amylase activity. Lipase was measured spectrophotometrically using a kit (Sigma Diagnostics, St Louis, USA). Two methods were used to measure IgG subclass concentrations: a nephelometric assay and a more sensitive ELISA; antisera were purchased from the Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands.

A Mono Q HR 5/5 anion exchange column (Pharmacia LKB, Uppsala, Sweden) was used to separate LDH isoenzymes. Plasma and concentrated dialysate samples were diluted three times with the equilibration buffer (50 mM tris/HCl, pH = 7.0); 1.0 ml was injected. Proteins were eluted from the column using a 0–500 mM NaCl gradient at pH 7.0 (flow = 1 ml/min). The eluate was collected in 40 0.5-ml fractions, in which LDH activity was measured by means of a lactate → pyruvate reaction (Technicon Chemicals Company, Tournai, Belgium). LDH activities are expressed in arbitrary units. At this pH, four peaks are detected, as both LDH 4 and LDH 5 appear in the void volume. The activity per peak was calculated by adding the activities in the fractions forming the peak. Total dextran 70 determination was done by means of high-performance liquid chromatography [15].

Cell lysates were made using the following protocol. A variable amount of dialysate was centrifuged (1200 r.p.m., 8 min.), yielding a clearly visible pellet. Cells were washed twice in NaCl 0.9%, resuspended in a lysis buffer (20 mM tris/ 150 mM NaCl/ 1% Triton/ 1 mM phenylmethylsulphonylfluoride/ 2 mM EDTA) and placed at 4°C for 30 min. Then the lysate was mixed vigorously and centrifuged; the LDH isoenzymes in the supernatant were separated and detected as described above.

The coefficients of variation of the protein assays were 5% for $\beta_2$ microglobulin, 10% for lysozyme, 6% for $\alpha_1$ microglobulin, 7% for lipase, 0.5% for pancreatic amylase, 0.7% for total amylase, 3% for albumin, transferrin, total IgG, and $\alpha_2$ macroglobulin, 2% for LDH, 10% for IgG subclasses when measured with ELISA and 3–6% when measured with nephelometry.

### *Calculations and data analysis*

The residual volume at the end of the dwell was calculated according to the indicator dilution method as described by Twardowski *et al.* [16], using dextran 70 as the indicator molecule [17]. Peritoneal clearances were calculated by multiplying the protein concentration in the effluent after 6 h with the effluent volume and dividing the product by the protein concentration in plasma and the dwell time. The effluent volume was calculated by adding the drained volume and the residual volume.

Results are given as mean ± SEM. Peritoneal clearances of the various proteins were compared using the Wilcoxon sign-

rank test. For the clearances of the LDH isoenzymes and the IgG subclasses this was done only when the test for repeated measurements indicated that significant differences were present. Data from stable and peritonitis patients were compared using the Mann–Whitney test. The clearances of the most neutral proteins ($\beta_2$ microglobulin, transferrin, total IgG, and $\alpha_2$ macroglobulin) were used to establish the size selectivity of the peritoneum in every patient. This was done by calculating the power relationship between the clearances of the above proteins and their molecular weight: $PC = a \times MW^s$, in which PC is the protein clearance; a is a constant and s, the size selectivity, is the slope of the linear regression line that is present when PC and MW are plotted on a double logarithmic scale [4]. The predicted peritoneal clearance of each of the proteins was calculated on the basis of this regression line. The differences between observed and predicted clearance were tested by a modified $t$ test [18]. This test takes the interindividual variability of the regression line into account.

## Results

Plasma and dialysate levels of all proteins in stable patients and during peritonitis are given in Table 1. As expected, plasma levels were not different between stable and peritonitis patients. Although all dialysate levels were higher in peritonitis patients than in stable patients, this difference was not statistically significant for $\beta_2$ microglobulin ($P=0.2$) and IgG4 ($P=0.13$), possibly (partly) due to the limited number of peritonitis patients. The peritoneal clearances of the proteins and their isoforms in the nine stable patients are given in Table 2. In Figure 1 the mean protein clearances are plotted against their molecular weight on a double logarithmic scale. The Pearson correlation coefficient (r) of the regression line was calculated in individual patients; the median was −0.986, range −0.968 to −0.999. All $P$ values of these correlations were below 0.05. Each protein clearance was compared with the clearance predicted on the basis of the molecular weight. Clearance of IgG3 was significantly lower than predicted ($P<0.01$); clearance of lipase ($P=0.05$) and LDH 4/5 ($P<0.01$) were significantly higher than

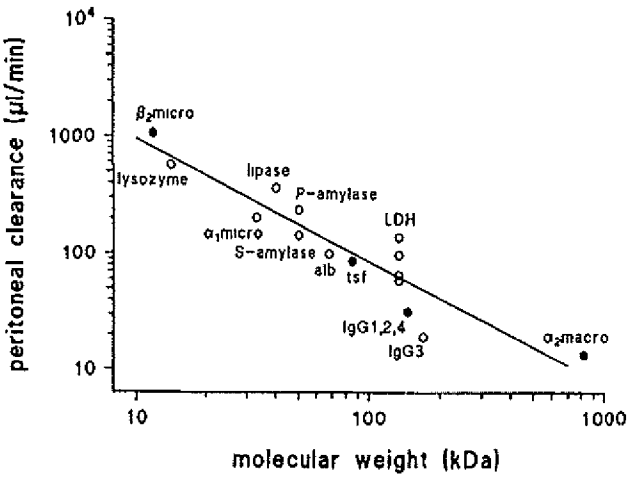


Fig. 1. The relationship between peritoneal clearance and molecular weight of plasma proteins in stable CAPD patients plotted on a double logarithmic scale. Mean values are given; $n=9$. Clearances of $\beta_2$ microglobulin ($\beta_2$micro), transferrin (tsf), total IgG, and $\alpha_2$ macroglobulin ($\alpha_2$macro) were used to draw the regression line (solid circles). Other abbreviations: $\alpha_1$micro, $\alpha_1$microglobulin; P-amylase, pancreatic amylase; S-amylase, salivary amylase; alb, albumin.

Table 1. Plasma and dialysate concentrations of plasma proteins (mean ± SEM) in nine stable CAPD patients and five CAPD patients during bacterial peritonitis

| Protein | Plasma | | Dialysate | |
|---|---|---|---|---|
| | Stable | Peritonitis | Stable | Peritonitis |
| $\beta_2$ microglobulin | 27.7±4.4 | 21,2±4.5 | 3.6±0.4 | 5.3±1.0 |
| Lysozyme | 4.9±1.0 | | 0.4±0.1 | |
| $\alpha_1$ microglobulin | 315±27.8 | | 8.4±1.5 | |
| Lipase* (U/l) | 50.5±10.1 | | 2.2±0.4 | |
| Pancreatic amylase* (U/l) | 123±26.2 | 203±104 | 2.9±0.3 | 13.3±4.4[a] |
| Salivary amylase* (U/l) | 158±27.5 | 85.9±13.2 | 2.6±0.4 | 4.6±0.8[d] |
| Albumin (g/l) | 34.8±1.8 | 27.4±3.2 | 0.5±0.07 | 1.0±0.3[b] |
| Transferrin | 2147±150 | 2246±518 | 25.4±4.2 | 78.5±19.2[b] |
| LDH 1* (arbitrary U/l) | 182±12.6 | 156±9.5 | 1.6±0.4 | 5.4±1.4[b] |
| LDH 2* (arbitrary U/l) | 345±18.9 | 305±21.3 | 3.1±0.7 | 17.9±4.7[a] |
| LDH 3* (arbitrary U/l) | 239±27.5 | 182±35.4 | 3.1±0.6 | 37.5±13.4[a] |
| LDH 4/5* (arbitrary U/l) | 135±17.1 | 160±55.9 | 2.7±0.7 | 92.4±38.8[a] |
| IgG1* | 7477±1076 | 6140±1864 | 34.0±5.4 | 196±51.9[a] |
| IgG2* | 1749±377 | 2484±1350 | 7.4±1.4 | 55.7±24.8[a] |
| IgG3* | 786±211 | 785±264 | 1.8±0.5 | 18.3±5.1[a] |
| IgG4* | 180±55.5 | 98.7±33.8 | 0.8±0.4 | 2.0±0.6 |
| IgG total* | 10450±1407 | 9141±1147 | 62.7±16.4 | 226±45.8[a] |
| $\alpha_2$ macroglobulin* | 1761±232 | 1402±121 | 4.0±1.6 | 18.1±6.9[c] |

IgG subclasses were measured by ELISA. Protein concentrations are expressed in mg/l unless otherwise specified.
[a] $P \leq 0.01$; [b] $P=0.02$; [c] $P=0.03$; [d] $P=0.05$ versus dialysate in stable patients.
*Protein concentration measured in concentrated dialysate; see text.

Table 2. Characteristics of plasma proteins (molecular weight in kDa and isoelectric point: $P_I$) and their peritoneal clearances (mean ± SEM, µl/min) in nine stable CAPD patients

| Protein | MW | $P_I$ | Peritoneal clearance | Significance |
|---|---|---|---|---|
| $\beta_2$ Microglobulin | 11.8 | 6.1 | 1072 ± 150 | a |
| Lysozyme | 14 | > 10 | 572 ± 69 | |
| $\alpha_1$ Microglobulin | 33 | 3.6–4.3 | 202 ± 43 | |
| Lipase | 40 | 4.7 | 362 ± 102 | |
| Pancr. amylase | 50 | 7.0 | 234 ± 97 | c |
| Saliv. amylase | 50 | 5.9–6.4 | 142 ± 35 | |
| Albumin | 68 | 4.7 | 99.0 ± 17.5 | a |
| Transferrin | 85 | 5.9 | 85.9 ± 16.5 | |
| LDH 1 | 134 | 5.2 | 58.5 ± 11.2 | b |
| LDH 2 | 134 | 5.9, 6.3 | 64.7 ± 16.1 | a |
| LDH 3 | 134 | 6.7 | 96.6 ± 19.0 | a |
| LDH 4/5 | 134 | 7.0–8.4 | 137.1 ± 27.9 | a |
| IgG1 | 146 | 8.0–9.5 | 32.0 ± 3.2 | a |
| IgG2 | 146 | 7.0–7.5 | 31.1 ± 3.9 | b |
| IgG3 | 170 | 8.2–9.0 | 19.1 ± 1.6 | a |
| IgG4 | 146 | < 6 | 31.2 ± 4.3 | |
| IgG total | | | 33.6 ± 5.0 | |
| $\alpha_2$ Macroglobulin | 820 | 5.4 | 13.5 ± 4.1 | |

All patients were studied during a 6-h dwell with glucose 2.27%. IgG subclasses were measured by ELISA.
[a] $P = 0.01$; [b] $P = 0.02$; [c] $P = 0.03$.

predicted. Clearances of all other proteins were within the range expected on the basis of their molecular weight ($P > 0.05$).

IgG1 clearance was 41.9 µl/min and IgG2 clearance was 42.5 µl/min ($P = 0.91$) measured with nephelometry. The clearance of IgG3 (MW = 170 kDa) was 31.4 µl/min ($P = 0.01$ vs IgG1 and vs IgG2). IgG4 clearance could not be calculated in six of the nine patients as the levels of IgG4 in concentrated dialysate were below the detection limit. Therefore we also measured IgG subclasses with ELISA. The clearances were somewhat lower than measured with nephelometry (Table 2). The test for repeated measurements indicated that no significant difference was present between the clearances of IgG1, IgG2, and IgG4; the clearance of IgG3 was significantly lower. The clearances of the LDH isoenzymes were higher with increasing $P_I$ (Table 2). The differences between the isoenzyme clearances were significant except for LDH 1 vs LDH 2 ($P = 0.41$). Transferrin clearance was 13% lower than albumin clearance. Pancreatic and salivary amylase differed significantly in clearance ($P = 0.034$). In two patients, salivary amylase was not detectable in plasma and/or dialysate; these patients were omitted from the analysis of amylase clearances. The peritoneal clearance of lipase, another pancreatic enzyme, was 362 µl/min and exceeded the clearance predicted on the basis of the regression line ($P = 0.05$). The clearance of the highly cationic lysozyme was 47% lower than that of $\beta_2$ microglobulin. $\alpha_1$ Microglobulin, a negatively charged protein, somewhat larger than the former two, showed a peritoneal clearance that was not different from the clearance predicted on the basis of its molecular weight ($P > 0.05$).

The peritoneal clearances of proteins during peritonitis episodes are shown in Table 3. The relationship with molecular weight is given in Figure 2. The median Pearson correlation coefficient between protein clearances and molecular weight in individual patients was

Table 3. Peritoneal clearances of plasma proteins (mean ± SEM, µl/min) in five CAPDpatients during bacterial peritonitis

| Protein | Peritoneal clearance |
|---|---|
| $\beta_2$-Microglobulin | 1091 ± 279 |
| Pancr. amylase | 503 ± 119 |
| Saliv. amylase | 302 ± 65 |
| Albumin | 211 ± 52 |
| Transferrin | 205 ± 55 |
| LDH 1 | 268 ± 72 |
| LDH 2 | 515 ± 163 |
| LDH 3 | 1772 ± 609 |
| LDH 4/5 | 4761 ± 1355 |
| IgG1 | 181 ± 43 |
| IgG2 | 144 ± 33 |
| IgG3 | 133 ± 37 |
| IgG4 | 117 ± 29 |
| $\alpha_2$-Macroglobulin | 68.0 ± 21.8 |

IgG subclasses were measured by ELISA.

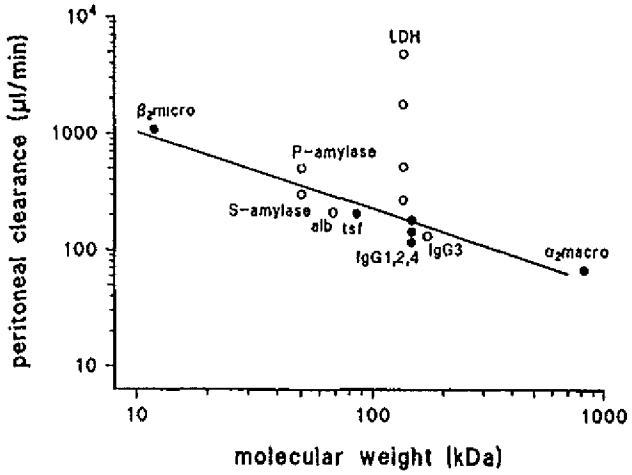


Fig. 2. The relationship between peritoneal clearance and molecular weight of plasma proteins in CAPD patients during bacterial peritonitis plotted on a double logarithmic scale. Mean values are given; $n=5$. See Fig. 1 for abbreviations.

$-0.988$, range $-0.941$ to $-0.999$; all $P$ values were below 0.06. The slope of the regression line was less than in the stable patients ($-0.67\pm0.08$ vs $-1.09\pm0.05$, $P<0.01$).

The LDH isoenzyme clearances in stable patients suggested charge selectivity of the peritoneal membrane. In peritonitis the peritoneal clearance of the most cationic LDH isoenzymes, especially LDH 4/5, showed a spectacular increase. It was more than 10 times the LDH 1 clearance, suggesting local release. The cell population present in the peritoneal effluent was studied as a potential source of local LDH release. The relative contributions of LDH 1, LDH 2, LDH 3, and LDH 4/5 to the total LDH content of the cells present in peritoneal effluent were calculated for five stable patients (Figure 3), left panel) and five peritonitis patients (right panel). Assuming that charge selectivity plays no role in transperitoneal transport, the relative amounts of LDH 2, 3 and 4/5 in dialysate can be calculated that are attributed to local release. In these calculations LDH 1 was assumed to reach the peritoneal cavity exclusively by transperitoneal transport as the amount of LDH 1 in the lysates of the effluent was very low (Figure 3), and as LDH 1 clearance was not different ($P>0.2$) from the LDH clearance predicted on the basis of the regression line. The part of the clearance of the other isoenzymes that exceeded the LDH 1 clearance was considered to be due to local release from cells. In Figure 3 the percentages of each of the LDH isoenzymes present in the lysates of cells in the effluent (open circles) are compared to the percentages of LDH 2, LDH 3, and LDH 4/5 in the dialysate that could be attributed to local release from cells (solid circles). In only one of the stable patients (left panel) LDH 3 and 4/5 contents differed largely in lysate and dialysate. During peritonitis the same pattern was seen as in the stable patients with regard to the distribution of the LDH isoenzymes in the lysed cells and in that of locally released LDH in the dialysate. It could be calculated from these data that a 22% lysis of the cells present in the effluent can account for the extra LDH clearance during peritonitis.

The albumin/transferrin clearance ratio was not different during peritonitis ($1.06\pm0.03$) compared to the stable situation ($1.17\pm0.04$, $P>0.05$). The same was found for the pancreatic/salivary amylase clearance ratio: $1.65\pm0.24$ during peritonitis and $1.48\pm0.19$ in the stable patients ($P>0.05$).

## Discussion

Power relationships have been described between the peritoneal clearances of plasma proteins and their molecular weights [4]. The slope of the linear regression line that was obtained when both parameters were plotted on a double logarithmic scale, indicated that

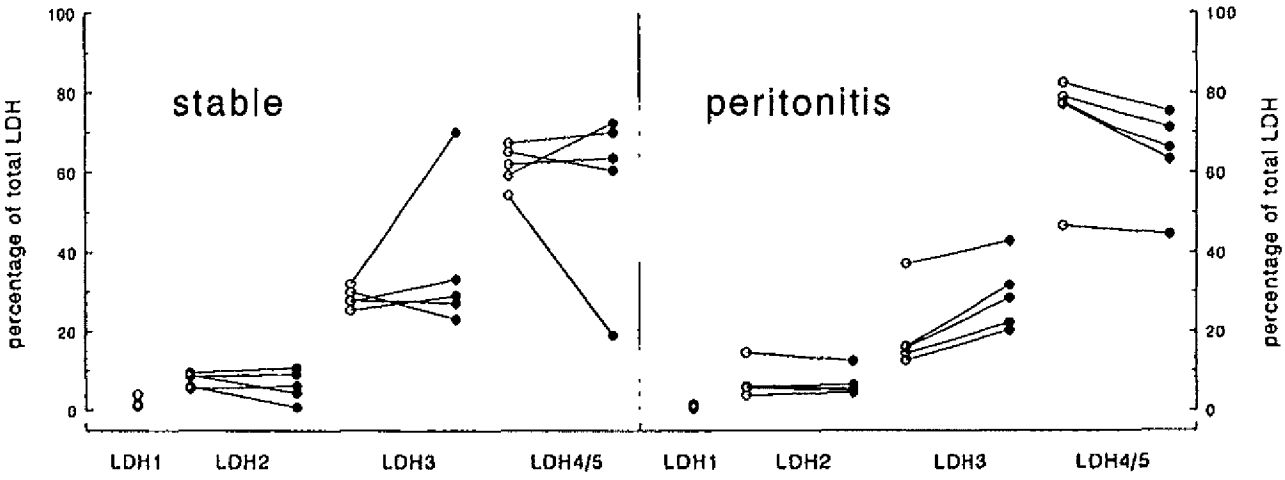


Fig. 3. LDH content of lysates of cells present in the peritoneal effluent (open circles) compared to the LDH isoenzymes in the dialysate of the same patient that are due to local release (solid circles), relative to total LDH. In these calculations it was assumed that the LDH 1 concentration in the dialysate is only dependent on transperitoneal transport (see text). Left: Stable CAPD patients ($n=5$). Right: Patients during bacterial peritonitis ($n=5$).

the peritoneal membrane constituted a size selective transport barrier to macromolecules. In the present study the regression line was drawn on the basis of the peritoneal clearances of proteins with isoelectric points close to neutrality. This enabled us to analyse the potential importance of electric charge in transperitoneal transport by comparing the observed clearances of charged proteins to the clearances predicted by the regression line and by comparing clearances of proteins with identical or almost identical size and shape but with different isoelectric points. During peritonitis, the slope of the regression line was less steep than in stable CAPD confirming older observations by our group [4].

In the present study we used the molecular weight as an estimate of protein size although the Einstein–Stokes radius or the free diffusion coefficient is often considered to be better [19]. However, the radius of only some of the proteins used in this study is known: $\beta_2$ microglobulin 16 Å, $\alpha_1$ microglobulin 28 Å, amylase 29 Å, albumin 36 Å, transferrin 40–43 Å, IgG 54 Å and $\alpha_2$ macroglobulin 89 Å. A linear relationship was present between the molecular weight and the radius of these proteins when plotted on a double logarithmic scale ($r=0.99$; $P=2.45\times10^{-5}$); none of the proteins deviated from this line. Therefore we considered the molecular weight an appropriate alternative, although we cannot rule out the possibility that one of the proteins with an unknown radius has a disproportionally small or large radius.

Clearances of highly anionic proteins such as albumin or $\alpha_1$ microglobulin and the clearance of the cationic lysozyme were not significantly different from the clearance expected on the basis of the molecular weight. Neither comparison of the clearance of transferrin with that of the negatively charged albumin, nor the clearance of $\beta_2$ microglobulin with that of the positively charged lysozyme provided evidence for charge selectivity of the peritoneal membrane. In both cases, the more cationic molecules (lysozyme, transferrin) appeared in the dialysate at a slower rate than the more anionic molecules. As lysozyme and transferrin have slightly greater molecular weights than their respective counterparts, their lower clearance can be attributed to the size selectivity of the peritoneal membrane. During peritonitis the relation between the clearances of albumin and transferrin appeared to be unaltered.

IgG subclasses measurement showed that no significant difference existed between the clearances of IgG1, IgG2 and IgG4, despite the differences in their isoelectric points. These results are in agreement with a previous pilot study in which no difference between IgG4/total-IgG ratios in serum and dialysate was found in five stable patients [9]. This shows that the peritoneal membrane does not retard the passage of the anionic IgG4 compared to the more cationic subclasses. The IgG3 clearance was lower than the clearances of the other subclasses and than the clearance predicted on the basis of its molecular weight. As IgG3 is cationic, charge selectivity of the peritoneal membrane cannot be held responsible. Both results can probably be explained by the higher molecular weight (170 kDa) and the three-dimensional configuration of IgG3 [20,21]. IgG3 has a large hinge region and therefore behaves as a protein with a disproportionally large radius compared to the other subclasses; in addition, the IgG3 molecule is especially susceptible to proteolysis.

LDH and amylase were the only proteins that suggested that the peritoneal membrane could act as a negatively charged barrier. In agreement with a study by Haraldsson [11], we observed that LDH isoenzyme clearances were higher with increasing isoelectric points in stable CAPD.

Gotloib *et al.* [7,8] showed in rats not treated with CAPD that the density of fixed negative charges was reduced during experimentally induced septic peritonitis. A reduction or disappearance of the differences between the LDH isoenzyme clearances in CAPD patients during an episode of peritonitis would therefore support the hypothesis that charge selectivity is responsible for the observed isoenzyme clearance pattern in stable patients. However, in five patients studied during bacterial peritonitis, an exceptional rise in the clearances of the most cationic isoenzymes was observed (LDH 4/5). This effect clearly cannot be explained by the charge selectivity hypothesis, and as the clearances of the cationic isoenzymes exceeded even the clearances of small proteins such as $\beta_2$ microglobulin, local release from cells was likely to occur. Lysis of the cells (predominantly macrophages and neutrophils) present in effluents of stable patients and during peritonitis, revealed an isoenzyme content that corresponded to the isoenzyme pattern of the locally released LDH in the dialysate. As this local LDH release mimics the effect of charge selectivity, the LDH clearance pattern observed in the nine stable patients is likely to be caused by release of LDH isoenzymes from the cells in the effluent and not by charge selectivity.

The difference between the clearances of the neutrally charged pancreatic amylase and the slightly anionic salivary amylase clearance also suggested charge selectivity of the peritoneum. However, during peritonitis the difference in their clearances remained unchanged. Therefore, the possibility of direct 'leakage' from the retroperitoneally situated pancreas was investigated. This was hypothesized because the peritoneal clearance of amylase is markedly increased during pancreatitis in CAPD patients [22]. This possibility was supported by the observation that the clearance of another pancreatic enzyme, lipase, was markedly higher than expected on the basis of its molecular weight.

Charged dextrans cannot be applied in humans. Leypoldt and Henderson [13] compared the peritoneal transport of neutral dextran, anionic dextran sulphate and cationic diethylaminoethyl dextran in rabbits. In a 4-h dwell it appeared that the transport of cationic dextran was markedly lower than that of the other dextrans, suggesting that the peritoneal membrane would behave as a positively charged barrier. The authors hypothesized that the peritoneal interstitium

could act as a cation exchanger and retard the passage of cationic macromolecules. This implies that when—after an equilibration period—the peritoneal interstitium is saturated with macromolecules, the clearances of positively and negatively charged and neutral dextrans can be expected to reach identical values. Plasma proteins are endogenous substances and therefore the peritoneal interstitium is saturated with them. As a consequence, the cation exchange properties of the interstitium are unlikely to play a role in clearance studies of endogenous proteins.

A study by Galdi *et al.* in rats [12] provided evidence, although indirect, pointing towards a negatively charged peritoneal barrier. These authors reported that the dialysate/plasma ratio of total protein increased after intraperitoneal administration of protamine, a polycation. The increase was not found when heparin, a polyanion, was added to the dialysate. According to the authors this increase was the consequence of a selective increase of the clearance of negatively charged proteins such as albumin, due to neutralization of anionic sites in the peritoneum by protamine. But positively and negatively charged proteins were not measured separately. Moreover, no dextran clearance study was performed after administration of protamine or protamine/heparin. Therefore other possible effects of protamine that influence the protein clearance, such as an increase in the peritoneal blood flow, cannot be excluded, and definite conclusions cannot be drawn from this study.

From studies in the glomerular basement membrane it is well known that negatively charged membranes retard the passage of anionic macromolecules [23]. We were unable, however, to demonstrate a charge selective barrier function of the peritoneum during CAPD. A possible explanation could be the continuous exposure of the peritoneal membrane to unphysiologically high glucose concentrations. This could lead to impairment of heparan sulphate metabolism [24] with loss of charge selectivity [25] as is the case in diabetic nephropathy. When this hypothesis is correct, charge selectivity should be present shortly after the initiation of CAPD. We were unable to demonstrate charge selectivity in two non-diabetic CAPD patients treated for 4 days and 1 month (data not shown). This however, needs, further confirmation.

In conclusion we were unable to demonstrate any effect of the isoelectric point of plasma proteins on their transport across the peritoneum during CAPD. LDH and amylase isoenzymes are not suitable for the assessment of transcapillary transport to the peritoneal cavity, because the former are influenced by cell lysis and the latter by direct leakage from the pancreas.

*Acknowledgements.* The authors acknowledge Dr A. M. Koster for statistical advice on the development of the modified *t*-test used to assess whether the clearance of a protein was different from the predicted value based on the size selectivity of the peritoneal membrane. Marianne E. M. Hansen-Hallewaart, Rudi de Waart, and Johan Hiralall are thanked for their excellent technical assistance.

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*Received for publication: 18.9.95*
*Accepted in revised form: 18.12.95*

Analytical Biochemistry
Volume 28, 1969, Pages 563-572

doi:10.1016/0003-2697(69)90210-3 | How to Cite or Link Using DOI



# Fractionation of fluorescent-labeled proteins according to the degree of labeling

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Received 18 October 1968. Available online 07 December 2004.





## Abstract

Labeling of macromolecules often facilitates the investigation of their properties and interactions. However, labeling normally leads to a heterogeneous population of labeled species which may differ significantly in their chemical and biological reactivity.

This report deals with a method for separating labeled materials into fractions according to their degrees of labeling.

Preparative acrylamide gel electrophoresis or gel filtration on Sephadex G-100 separate fluorescent-labeled proteins into fractions having different degrees of labeling.

The dansyl and the fluorescein derivatives of ovalbumin and bovine serum albumin have been studied. Typical electrophoretic separations result in fractions differing by as little as 0.05–0.1 in the average mole ratio of dye to protein. However, the nonintegral values obtained make it clear that the preparations are still nonuniform with respect to degree of labeling. Fractions obtained by gel filtration show several bands when tested by analytical acrylamide gel electrophoresis, while those obtained by preparative gel electrophoresis show only a single band.

In the electrophoretic separations, the more rapidly migrating species were found to be more heavily labeled. This finding is explicable on the basis that the introduction of a dye molecule onto a protein should alter the net charge by −3 for fluorescein, and by −1 for dansyl.

## Article Outline



Analytical Biochemistry
Volume 28, 1969, Pages 563-572